Influence of Incubation pH on *in vivo* Nitrate Reductase Activity in Leaves of Two Perennial and Two Annual Tropical Plants

C. C. Subbaiah and P. Manikandan

Central Plantation Crops Research Institute (CPCRI), Regional Station, Vittal-574 243, Karnataka, India

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Summary

The effect of assay medium pH on *in vivo* nitrate reductase (NR) activity of cashew (*Anacardium occidentale* L.) leaves was studied. The enzyme activity continued to increase beyond pH 7.5 up to pH 10 until the leaf discs showed symptoms of damage. Entry of nitrate into the leaf tissue also increased up to pH 9.

Higher activities of NR at alkaline pH were also observed in cacao, country bean (*Dolichos lablab*) and sugarcane leaves. This is perhaps the first time that such a high range is reported as optimal pH for NR in higher plants.

Key words: Anacardium occidentale, Dolichos lablab, Saccharum officinarum, Theobroma cacao, incubation pH, nitrate reductase activity.

Introduction

Nitrate reductase (NADH: nitrate oxidoreductase EC 1.6.6.1) which reduces nitrate to nitrite is a key enzyme in plant nitrogen metabolism. The enzyme can be assayed by an *in vivo* method (Jaworski, 1971; Radin and Sell, 1975) and the activity estimated by this method correlates well with extractable NR activity (Streeter and Bosler, 1972). This assay system depends on the absorption of nitrate, endogenous generation of NADH and subsequent release of nitrite into the medium (Srinivasan et al., 1982). Hence the activity is influenced by the assay conditions (Srinivasan et al., 1982; Subbaiah, 1982) besides the metabolic status of the plant.

In an attempt to characterise the assay system for this enzyme in cashew, we found that the optimal pH of the incubation medium for NR activity in this species differed from the pH range (7–7.5) reported in the literature for other plants (Jaworski, 1971; Campbell, 1976). Subsequently we checked the enzyme response in three more

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species, viz., cacao, country bean and sugarcane. The pH of the medium during infiltration and incubation had significant effect on the *in vivo* nitrate reductase assay in all the species studied.

Materials and Methods

Plant material: Cashew (*Anacardium occidentale* L.) and cacao (*Theobroma cacao* L. cv. Forestero) leaf samples were obtained from field grown trees at CPCRI, Vittal (12° 52″ latitude, 75° E longitude and 200 m altitude). Soil is lateritic with a pH of 5 to 5.7 and water holding capacity of 30–40 per cent in the top 25 cm of the soil. The studies were made during 1981–82 and sampling was done both in the dry and wet seasons. Young fully expanded leaves were collected generally between 10–11 h.

Cashew trees were about 9 years old raised from seedlings, spaced at 8 × 8 m and were fertilized at the rate of 250 : 125 : 125 g ($N : P_2O_5 : K_2O$) in two split doses. The trees were not irrigated. Cacao was grown as a mixed crop in the interspaces of arecanut (*Areca catechu* L.) spaced at 4 × 4 m. The trees were fertilized at the rate of 100 : 40 : 140 g ($N : P_2O_5 : K_2O$) and given summer irrigation.

Country bean [*Dolichos lablab* L.; syn. *Lablab purpureus* (L.) Sweet] and sugarcane (*S. officinarum* L.) samples were collected from homestead gardens of a nearby area.

Enzyme assay: NR activity was assayed essentially by the *in vivo* method of Jaworski (1971). 0.2 g of tissue was infiltrated with 2.5 ml of 0.1 M potassium phosphate buffer and 2.5 ml of 0.2 M KNO_3. Buffers of pH 2, 4, 6, 7.5, 8, 9, 10 and 11 were used and the resultant pH of the incubation media were 2.18, 4.40, 5.97, 7.46, 7.97, 8.74, 9.37 and 10.58 respectively. The incubation was done for 30 min and nitrite was estimated colorimetrically (Jaworski, 1971).

In the case of cashew, the leaves were sliced under water (Subbaiah, 1982) blotted dry and 0.5 g of this tissue was used for the assay with an incubation period of 1 h. The medium contained 0.01 % sodium diethyl dithiocarbamate (Subbaiah, 1982) besides the above components (Jaworski, 1971).

As boiling has been reported to result in the loss of nitrite at acidic pH (Srinivasan et al., 1982), the leaf slices were killed by 3 N HCl used in the preparation of sulfanilamide reagent. This facilitated the gradual release of nitrite into the medium at room temperature. Alternatively, the medium was made alkaline at the end of incubation period by adding 2 % KOH (Srinivasan et al., 1982) whenever boiling was done. Wherever necessary, the incubation mixture was clarified before colour development, using activated charcoal.

Nitrate estimation: Besides estimating nitrite formation, disappearence of nitrate from the external medium was also monitored in assays with cashew. Nitrate was assayed according to Cataldo et al. (1975) with the following modifications:

The solutions were decolourised using activated charcoal before taking aliquots for nitrate determination and excess of NaOH was added to completely solubilize the nitrosalicylic acid complex.

Results and Discussion

The influence of external pH on NR activity of cashew leaves is represented in Fig. 1. The enzyme activity increased significantly up to pH 7.5 and thereafter the upward trend was gradual. The leaf slices were damaged at or beyond pH 10 as indicated by the release of phenols and heavy browning of the assay medium. In subsequent experiments the leaf slices were pre-incubated in buffers of different pH for 1–2 h, washed and then used for assay. The assay was done at neutral pH but still the

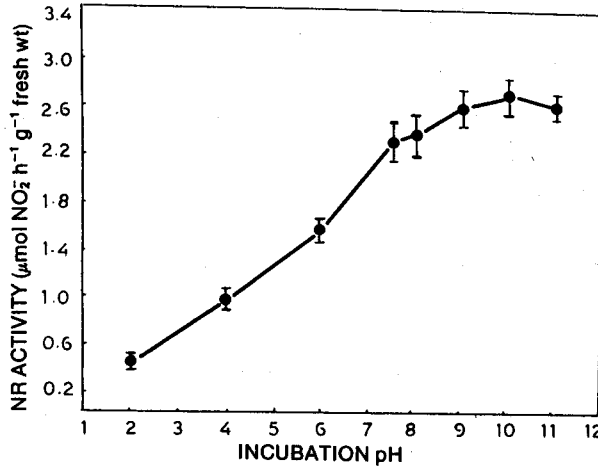

Fig. 1: Effect of infiltration and incubation pH on *in vivo* nitrate reductase activity of cashew. Boiling was avoided by killing the leaf tissue on sulfanilamide-HCl addition. The values are averages ± S.E. of more than 10 experiments. Critical difference (P = 0.05) is 0.36.

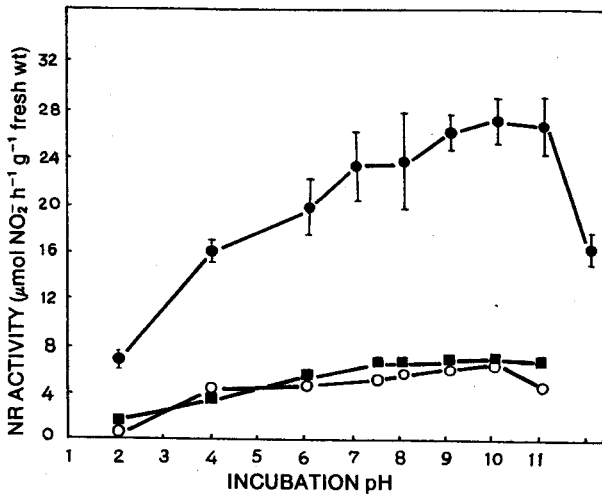

Fig. 2: Effect of incubation pH on *in vivo* NR activity of cacao (——●——), bean (——○——) and sugarcane (——■——). The values are the means ± S.E. of three experiments in cacao. The data on bean and sugarcane represent mean values of 2 replicates.

enzyme response to pre-incubation pH did not differ much from the above pattern. We do not know whether the enzyme was irreversibly affected at higher pH.

Levels of Endogenous Cytokinin-like Substances in *Rosa rugosa* Achenes During Dormancy Release and Early Germination

Åsa Julin-Tegelman

Department of Botany, Section of Plant Physiology, University of Stockholm, 106 91 Stockholm, Sweden

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Summary

Rosa rugosa var. rubra possess not only a coat-induced dormancy but are also embryo dormant and need a period of chilling to germinate.

Achenes stored under moist, cold conditions, showed marked but transient peaks of free and bound cytokinin-like substances detected by the Amaranthus test. In achenes stored at 17 °C no such changes were observed. Germinating achenes, previously stored at 4 °C for 14 weeks, showed a transient peak of bound cytokinin-like substances and no changes in free forms. The early changes in cytokinin-like substances during cold stratification are considered to be related to the breakage of dormancy while the second increase in cytokinins seems to be more closely involved with germination. Most of the cytokinin-like activity detected in the Amaranthus test co-chromatographed with zeatin and ribosylzeatin on paper-chromatography. Scarification of achenes with 70% sulphuric acid did not improve germination or reduce the time needed for chilling. The germination was, however, increased two-fold in partly after-ripened achenes by removing leached inhibitors from the imbibition water. The role of the seed coat is discussed.

Key words: Rosa rugosa var. rubra, Amaranthus-test, chilling, cytokinins, dormancy, germination.

Introduction

Although there are many reports dealing with hormonal changes during either dormancy release or germination, only a few have investigated the hormonal changes during both these processes in the same species.

In species which require a period of chilling to break dormancy it has been shown that cytokinin-like activity fluctuated markedly during cold stratification (Van Staden et al., 1972; Webb et al., 1973; Brown and Van Staden, 1973; Borkowska and Rudnicki, 1975; Tomaszewska, 1976; El-Antably, 1976; Pinfield and Davies, 1978; Dungey et al., 1980; Julin-Tegelman and Pinfield, 1982). Few workers however, have studied cytokinin changes during the germination process which follows an inductive cold period. Much of the work conducted on this aspect has involved *Acer* species. The reports in the literature do however, contain contradictory results. For instance,

Abbreviations: ABA = abscisic acid, S.E. = standard error of mean.

The pH effect was further confirmed by varying the buffer composition and thus excluding any specific ion effect. Citrate (sodium citrate – citric acid) buffer was employed for acidic pH and carbonate (sodium carbonate – bicarbonate) buffer for alkaline ranges. However, NR activity maintained an upward trend with increasing pH irrespective of the composition of the buffer. Again, pH values above 10 were injurious to cashew leaves in this case too.

Cacao and bean leaves also showed optimal enzyme activity at pH 9–10 (Fig. 2). Though sugarcane too showed high NR at alkaline pH, the activity did not increase any further beyond pH 7.5 (Fig. 2). In all the four species tested, NR activity showed a sharp decline below pH 7.5, similar to the response obtained in several other plant species (Jaworski, 1971; Knypl, 1973). It must be emphasised that this effect of pH could not be attributed to any nitrite loss while boiling at acidic pH as reported in the case of wheat (Srinivasan et al., 1982), since the pH-profile of enzyme activity remained the same even when precautions were taken against any possible nitrite loss. Below pH 7.5 considerable nitrite was lost due to boiling, but nevertheless the enzyme activity was consistently lower at this pH range even when alkalinization was done before boiling (Table 1). Hence the statement (Srinivasan et al., 1982) that there is no effect of external pH on *in vivo* assay of NR may not be valid in all cases.

Table 1: Effect of incubation pH and prevention of nitrite loss during boiling on NR activity in cashew.
After infiltration and 1 h incubation at different pH values, the pH was raised by the addition of KOH before boiling. Charcoal clarification was necessary before colour development (to remove the phenols released during boiling). The values are percentages of the activity at pH 9 (16.5 ± 1.1 μmol NO_2^- h^{-1} g^{-1} dry wt.) which was the maximum. The experiment is replicated 3 times and data are the means ± S.E.

Incubation pH	NRA	
	−KOH	+KOH
2	1.1±0.2	3.9± 0.3
4	6.1±1.3	9.7± 2.4
6	13.2±1.3	19.6± 5.7
7.5	47.1±5.7	50.8±10.3
9	100.0	–
10	95.0±1.9	–
11	71.5±6.7	–

Such an increased enzyme activity at alkaline pH as observed in this experiment has so far been reported only in the case of cyanobacteria, *Anacystis nidulans* (Manzano et al., 1976) and *Nostoc calcicola* (Kashyap et al., 1982). In *Anacystis*, the optimum pH for *in vitro* activity of ferredoxin-dependent NR was 10.5 and was pH 9 in the case of *Nostoc*. The pH optimum in many higher plants was 7 to 8 for *in vitro* NR activity (Knypl, 1973; Mendel and Muller, 1980) and reports are scanty as far as pH effect on *in vivo* activity is concerned (Jaworski, 1971; Shrinivasan et al., 1982).

Though intracellular pH is not much altered by external pH (Raven and Smith, 1980), it may be speculated that the high pH of incubation medium would influence the permeability of the plasmalemma and stimulate entry of NO_3^- into the leaf tissue. In fact, the uptake of nitrate from the medium was found to increase along with the pH of incubation mixture (Fig. 3). However, it is not certain whether stimulated NO_3^- uptake was the cause or result of enhanced NR activity at high pH. Alternatively the slight intracellular alkalinity that could be induced by that of the medium may activate the enzymes involved in the generation of NADH (Viz., enzymes of citric acid cycle). Thus, the enhanced availability of the substrate or reducing potential or both might have promoted the enzyme activity at high pH.

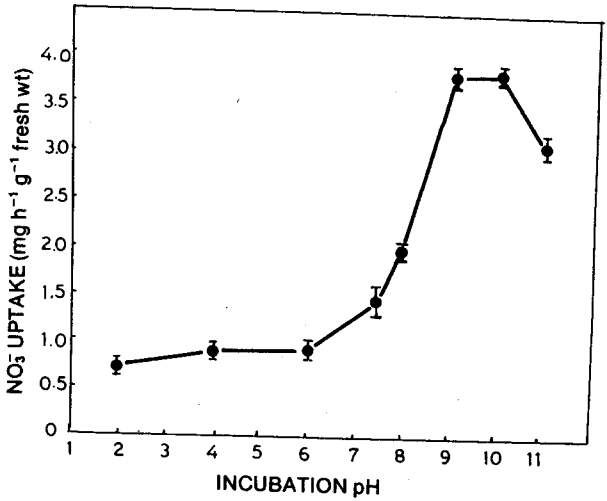

Fig. 3: Effect of infiltration and incubation pH on nitrate uptake in cashew. Leaf discs were incubated at different pH values and nitrate uptake was determined. The values are averages ± S.E. of more than ten experiments. Critical difference (P = 0.05) is 0.07.

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Recently Mengel et al. [MENGEL, K., P. ROBIN, and L. SALSAC: Plant Physiol. *71*, 618–622 (1983)] have reported a similar response of NR activity in maize, to the pH of nutrient solutions and attributed this to an increased synthesis of organic anions at alkaline pH ranges.